Chinese Herbs (Lonicera japonica and Ganoderma lucidum) Enhance Non-Specific Immune Response of Tilapia, Oreochromis niloticus, and Protection Against Aeromonas hydrophila

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ABSTRACT

Three diet variations, in addition to control (no herbs), were used to determine the effect of Chinese herbs (Lonicera japonica and Ganoderma lucidum) on non-specific immune response of tilapia. These contained 1.0% of Lonicera, 1.0% of Ganoderma and a mixture of Ganoderma (0.5%) and Lonicera (0.5%). The diets were fed for 3 weeks. The respiratory burst activity of blood leukocytes, phagocytosis, plasma lysozyme, total protein and total immunoglobulin were monitored. Following three weeks after feeding, fish were infected with Aeromonas hydrophila and mortalities recorded. The results of this study showed that feeding tilapia with Ganoderma and Lonicera alone or in combination enhanced phagocytosis by blood phagocytic cells during the whole experimental period and stimulated lysozyme activity after two weeks. Respiratory burst activity of phagocytic blood cells, total protein and total immunoglobulin in plasma were not enhanced. Both herbs when used alone or in combination increased the survival of fish after challenge with A. hydrophila. The highest mortality was observed in control fish – 58% and fish fed with Lonicera extract – 43%, while 30% of fish died in the group fed with Ganoderma and the lowest mortality (21%) was observed when fish were fed with a combination of two herbs. Thus, it can be concluded that the herb extracts added to diets acted as immunostimulants and appeared to improve the immune status and disease resistance of fish.


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INTRODUCTION

Diseases caused by *Aeromonas hydrophila* are some of the most widespread in freshwater fish culture. Septicaemia caused by motile aeromonads is a ubiquitous problem that affects fishes found in warm, cool, and cold fresh water around the world (Plumb, 1999). The bacterium, *A. hydrophila* has been associated with disease in carp, eels, milkfish, channel catfish, tilapia and ayu and can also be an opportunist in stress-related diseases in salmonids (Miyazaki and Jo, 1985; Rahman *et al.*, 1997; Plumb, 1999). Antibiotics are frequently used to control disease caused by this bacteria, but there is an increasing risk of developing antibiotic resistant strains of bacteria. Vaccines are being developed against *A. hydrophila* and atypical *A. salmonicida* but these are not yet commercially available and as *A. hydrophila* is such a heterogeneous species (multiple strains), vaccine development is extremely complex.

Our research is directed in an alternative, promising area. Herbs can act as immunostimulants, conferring early activation to the non-specific defense mechanisms of fish and elevating the specific immune response. Herbs have been used as medicine and an immune booster for humans for thousands of years in China. Recently, a growing interest has developed in using herbs in animal feeds by both researchers and feed companies. Chinese herbs contain many immunologically active components such as polysaccharides, organic acids, alkaloids, glycosides and volatile oils, which can enhance immune functions. Chinese herbs have been used as medicine to treat different fish diseases in China for many years. Herbs have also been used in the other countries for control of shrimp and fish disease, and successful results have been reported in Mexico, India, Thailand and Japan (Auro de Ocampo, 1993; Dey and Chandra, 1995; Direkbusarakom, 1996; Logambal and Michael, 2000). Recently, there has been increased interest in the immune stimulating function of some herbs in aquaculture. The non specific immune functions such as bacteriolytic activity and leukocyte function were improved by some mixtures of Chinese herbs in shrimp (*Penaeus chinensis*) and tilapia (Luo, 1997; Chansue *et al.*, 2000).

Two herbs were selected for the current study: *Lonicera japonica* and *Ganoderma lucidium*. Tilapia (*Oreochromis niloticus*) were fed with the extracts of Chinese herbs either alone or in combination for three weeks to investigate the effect of these substances on the non-specific immune response of tilapia and to examine protection levels against a challenge by *A. hydrophila*.

MATERIALS AND METHODS

Fish

Tilapia (*O. niloticus*) (52.5±3.50g) were acclimatised in a recirculation system at the Research Institute for Fisheries, Aquaculture and Irrigation (HAKI, Szarvas, Hungary). Fish were fed with a dry feed, produced in the experimental milling facility of the institute and kept in 2000 l fibreglass tanks with water temperature maintained between 22-23°C.
Herbal extracts

*Lonicera* extract containing 25% of chlorogenic acid, and *Ganoderma* extract containing 30% of *Ganoderma* polysaccharide were commercial products obtained from Xuancheng Baicao Plants Industry and Trade Ltd. China.

Experimental design and sampling procedure

Experiments were performed in the recirculation system of HAKI, Szarvas, Hungary. Batches of three-month old tilapia with an average initial weight of 52.5±3.50 g were held in 100 l fibreglass tanks. Fish were divided onto 8 groups, each group contained 60 fish. Water temperature and pH were constant (22-23°C; pH 8.5) during the experimental period, and dissolved oxygen was maintained at 80-90% of saturation. Water flow was maintained at 7 l/min.

Fish were fed *ad libitum* 6 times daily with the appropriate pelleted feed using an automatic feeder. Three diet variations, in addition to control (no herbs), were used. These contained 1.0% of Lonicera, 1.0% of *Ganoderma* and a mixture of *Ganoderma* (0.5%) and *Lonicera* (0.5%). Each group was fed in duplicate. The diets were fed for 3 weeks. Blood samples (6 fish/group) were collected from caudal vein one, two and three weeks after start of feeding. Heparin was used as an anticoagulant. Individual fish were sampled only once to avoid any influence on the assays due to multiple bleeding and handling stress on the fish.

Separation of leukocytes from the blood

Leukocytes for assay were separated from each blood sample by density-gradient centrifugation. One ml of histopaque 1.119 (Sigma) containing 100 µl of bacto hemagglutination buffer, pH 7.3 (Difco, USA) was dispensed into siliconised tubes. One ml of a mixture of 1.077 density histopaque and hemagglutination buffer and 1 ml of blood was carefully layered on the top. The sample preparations were centrifuged at 700 g for 15 min at 4°C. After centrifugation, plasma was collected and stored at -80°C for future analysis. Separated leukocytes were gently removed and dispensed into siliconised tubes, containing phenol red free Hanks Balanced Salt Solution (HBSS, Sigma). Cells were then washed twice in HBSS and adjusted to 2x10⁶ viable cells/ml.

Respiratory burst activity

Respiratory burst activity of isolated leukocytes was quantified by reduction of ferricytochrome c (Secombes, 1990). Briefly, 100 µl of leukocyte suspension and an equal volume of cytochrome C (2 mg/l in phenol red free HBSS) containing phorbol 12-myristate 13-acetate (PMA, Sigma) at 1 µg/ml were placed in triplicate in microtiter plates. To test specificity another 100 µl of leukocyte suspension and solutions of cytochrome C containing PMA and superoxide dismutase (SOD, Sigma) at 300 U/ml were prepared in triplicate in microtiter plates. Samples were then mixed and incubated at room temperature
for 15 min. Extinctions were measured at 550 nm against a cytochrome C blank in a
multiscan spectrophotometer. Readings were converted to nmoles $O_2^-$ by subtracting the
O.D. of the PMA/SOD treated supernatant from that treated with PMA given alone for
each fish, and converting O.D. to nmoles $O_2^-$ by multiplying by 15.87. Final results were
expressed as nmoles $O_2^-$ produced per $10^5$ blood leukocytes.

**Phagocytosis assay**

Phagocytosis activity of blood leukocytes was determined spectrophotometrically by
the method of Seely *et al.*, (1990). This assay involves the measurement of congo red-
stained yeast cells that have been phagocytosed by cells. To perform the assay, 250 µl of
the leukocyte solution was mixed with 500 µl of the congo red-stained and autoclaved
yeast cell suspension (providing a yeast cell: leukocyte ratio of 40:1). The mixtures were
incubated at room temperature for 60 min. Following incubation, 1 ml of ice-cold HBSS
was added and one ml of percoll (1.055) was injected into the bottom of each sample
tube. The samples were centrifuged at 850 g for 5 min to separate leukocytes from free
yeast cells. Leukocytes were harvested and washed twice in HBSS. The cells were then
resuspended in 1 ml trypsin-EDTA solution (5.0 g/l trypsin and 2.0 g/l EDTA, Sigma) and
incubated at 37°C overnight. Absorbance of the samples was measured at 510 nm using
tryptsin-EDTA as a blank.

**Lysozyme assay**

Plasma lysozyme activity was measured spectrophotometrically by the method of
Sankaran and Gurnani (1972). The lysozyme substrate was a 0.02 % (w/v) suspension
of Micrococcus lysodeikticus in phosphate buffer (0.05 M, pH 6.2). Lyophilised hen egg
white lysozyme was used as a standard. A new standard curve was prepared for each assay.
Standard solutions as well as samples were added to the substrate at 25°C. The results were
expressed as mg/ml equivalent of hen egg white enzyme activity.

**Total protein**

Plasma total protein was measured by the biuret method using a commercially available
kit (Reanal, Hungary)

**Total immunoglobulin**

Plasma total immunoglobulin was measured using the method described by Siwicki *et
al.*, (1994). Analysis of total immunoglobulin in plasma is based on the biuret method.
However, primary separation of immunoglobulins from the plasma was achieved by
precipitation with polyethylene glycol (PEG) and the resulting supernatant analysed. To
perform the assay 100 µl of the plasma was combined with 100 µl 12% PEG and incubated
at room temperature for 2 hr while being continuously mixed. Following incubation the
mixture was centrifuge for 10 min at 400 g and total protein concentration in the supernatant
was determined by the biuret method. Total immunoglobulin value for individuals was
calculated from total protein value less the quantity of protein in the supernatant.
Bacterial challenge

Groups of 30 fish from each treatment were challenged by intraperitoneal injection after three weeks of feeding. *A. hydrophila* (strain B-02/12 Bacteriology Laboratory, Institute of Aquaculture University of Stirling, Scotland), isolated in Bangladesh was used as the challenge strain. The bacteria were cultured in tryptone soya broth (TSB) overnight at 28°C, washed twice with sterile phosphate buffered saline (PBS) and then resuspended in PBS. The concentration of the suspension was determined spectrophotometrically using a pre-made standard curve relating concentration to absorbance at 610 nm. The concentration of bacterial suspension was adjusted accordingly with sterile PBS, prior to performing challenges.

A preliminary challenge using an unrelated, but similar, group of tilapia was performed to establish the LD$_{50}$ dose of the bacterium prior to performing the challenge of fish under study. The LD$_{50}$ was found to be 3 x 10$^6$ cells/ml and was used at this rate for the subsequent challenge. Fish were injected intraperitoneally (i.p.) with 0.1 ml of the bacterial suspension. Mortalities were observed for 10 days. Bacterial swabs were taken from the kidneys. All the surviving fish were killed using an overdose of anaesthetic.

Statistics

Results are presented as the average (± standard error) for five fish, and were compared at each time point using one way ANOVA and Dunn’s multiple range tests (Sigma Stat 3.2). Significant differences between experimental groups were expressed at a significance level of $P<0.05$.

RESULTS

The non-specific defence mechanism values for the fish fed with herb extracts are shown in Figures 1-5. Phagocytic activities of leukocytes were elevated for the whole period of the experiment and each treatment showed values higher than untreated control, the highest value was measured on week three in the group fed a combination of herbs (Figure 1). Herb supplementation in tilapia had no effect on respiratory burst activities of isolated phagocytic cells for the duration of the experiment. The production of oxidative radicals measured during 3 weeks of feeding the fish with all diets remained on the same level (Figure 2). The plasma lysozyme activities were significantly higher after two weeks in groups fed with herb extracts. However, there were no significant differences between treated groups (Figure 3). There were no significant changes in plasma protein (Figure 4) and total immunoglobulin during the whole experiment (Figure 5).

The results from the virulent pathogen challenges are shown in Figure 6. The highest mortality was observed in control fish (~58%) and fish fed with *Lonicera* extract (~43%), while 30% of fish died in the group fed with *Ganoderma* and the lowest mortality (21%) was observed when fish were fed with a combination of two herbs.
Figure 1. Phagocytic activity of isolated phagocytic cells in tilapia in control group and groups fed diets containing different kind of herbs. Data is expressed as the mean of six fish±SEM. Significance differences (P<0.05) from the untreated control are indicated by asterisks. Significant differences among group are indicated by letters.

Figure 2. Changes in respiratory burst activities of phagocytic cells isolated from blood in tilapia in control group and groups fed diets containing different kind of herbs. Legends are the same as in Figure 1.
Figure 3. Changes in plasma lyzosyme activities in tilapia in control group and in groups fed diets containing different kind of herbs. Legends are the same as in Figure 1.

Figure 4. Changes in plasma plasma total protein values in tilapia in control group and in groups fed diets containing different kind of herbs. Legends are the same as in Figure 1.
**Figure 5.** Changes in plasma total immunoglobulin values in tilapia in control group and in groups fed diets containing different kind of herbs. Legends are the same as in Figure 1.

**Figure 6.** Cumulative mortalities (%) of tilapia in control and in groups fed diets containing different kind of herbs and over a 6 days after artificial challenging with *Aeromonas hydrophila*. Thirty fish from each group were used.
**DISCUSSION**

In this study, extracts of two Chinese herbs from Ganoderma (*Ganoderma lucidium*) and from *Lonicera* (*Lonicera japonica*) were chosen because of their recorded ability to enhance the activity of the immune system. *Ganoderma lucidium* is a traditional Chinese medicine used for the prevention and treatment of various human diseases in China and other Asian countries (Lin, 2001). It has been shown that an aqueous extract from *G. lucidum* will promote phagocytosis by macrophages in mice immunosuppressed by cyclophosphamide (Tang, 2000; Wang *et al.*, 2003), it will stimulate proliferation of lymphocytes induced by concanavalin A or lipopolysaccharide and influence gene expression of cytokines (Wang *et al.*, 1997). The second herb, *L. japonica* has been known as an anti-inflammatory agent and used widely for upper respiratory tract infections, diabetes mellitus and rheumatoid arthritis (Lee *et al.*, 1998). It has been reported that *Lonicera* significantly increased blood neutrophil activity and promoted phagocytosis by the neutrophils in bovine at the correct concentration (Hu *et al.*, 1992).

The results from this study showed that both *Ganoderma* and *Lonicera* were able to modulate some parameters of the innate immune system of tilapia. The non-specific defence mechanisms of fishes include neutrophil activation, production of peroxidase and oxidative radicals, together with initiation of other inflammatory factors (Ellis, 1977; Ainsworth *et al.*, 1991). In this study fish fed with herb extracts showed an elevated phagocytosis over the whole period of the experiment. Fishes treated with immunostimulants usually show enhanced phagocytosis. Several studies have reported that oral administration of yeast products (MacroGard; Vitastim; *Saccharomyces cerevisiae*), (Aisworth *et al.*, 1994; Siwicki *et al.*, 1994; Jeney *et al.*, 1997), chitin (Sakai *et al.*, 1992), plant extracts, such as ginger increased the phagocytic capability of the cells in rainbow trout (Dügenci *et al.*, 2003) and extracts of four Chinese herbs (*Rheum officinale*, *Andrographis paniculata*, *Isatis indigotica*, *Lonicera japonica*) increased phagocytosis of white blood cells of crucian carp (Chen *et al.*, 2003).

Total lysozyme is a measurable humoral component of the non-specific defence mechanism, and while reports on modulation of the lysozyme activity in fishes are rare, increased values have been recorded by various authors after activation of the immune system with immunomodulants (Engstadt and Robertsen., 1993; Siwicki *et al.*, 1994; Thompson *et al.*, 1995) and by feeding four different Chinese herbs (*Rheum officinale*, *Andrographis paniculata*, *Isatis indigotica* *Lonicera japonica*) (Chen *et al.*, 2003). In our previous study it was shown, that Astragalus enhanced lysozyme activities in tilapia during the whole period of the experiment when fed low (0.1%) and medium (0.5%) doses of herbs, while no effect was found when fish were fed with Scutellaria (Yin *et al.*, 2006).

In this study we used the reduction of ferricytochrome c to determine extracellular superoxide anion. Jeney *et al.*, (1997) showed that extracellular activity was very high in fish fed with dietary glucan. Rainbow trout fed with ginger (*Zingiber officinale*) extract had significantly higher extracellular activity of phagocytic cells in blood (Dügenci *et al.*, 2003). However, in this study we could not detect differences in respiratory burst activity in fish nor in our previous studies, when the tilapia were fed with Astragalus extract. Respiratory burst activity of phagocytic cells was not elevated and in the case of
fish fed with Scutellaria there was significant inhibition of extracellular superoxide anion production (Yin et al., 2006). It was shown that in trout fed with nettle and mistletoe extracts the production of extracellular superoxide anion was of a similar level to that in the control fish (Dügenci et al., 2003).

Total protein level and total immunoglobulin was not affected by feeding tilapia with herbs. Immunostimulants usually do not affect total protein levels, but fish with nutritional deficiencies may show lower plasma total protein levels (Siwicki et al., 1994). On the other hand a decrease of total protein level in Russian sturgeon plasma was found after treatment of fish with glucan, chitosan and finnstim by immersion (Kolman et al., 1998). Immunostimulants may raise the total immunoglobulin levels. Sturgeon treated with epin by immersion showed a higher concentration of total immunoglobulin (Kolman, 2001), whereas when chitosan, glucan and finnstim were administered by immersion the level of total immunoglobulin was decreased (Kolman et al., 1998).

After challenge with A. hydrophila survival of fish fed with herb extracts was improved when compared with the control group. The survival was further enhanced in the group fed the Ganoderma extract supplement and when both herbs were used together. It is possible that this is the result of enhancement of some components of non-specific immune system of the fish by Ganoderma and a combination of Ganoderma and Lonicera. There is strong evidence that feeding glucans can modify the activity of the innate immune system of fish and increase the disease resistance in several fish species (Anderson, 1992; Sakai, 1999; Raa, 2000). Feeding carp with chitosan (1%) and levamisole (250 mg/kg of diet) also increased survival of fish following challenge with A. hydrophila (Gopalakannan and Arul, 2006).

The results of this study showed that feeding tilapia with Ganoderma and Lonicera alone or in combination enhanced phagocytosis by blood phagocytic cells during the whole experimental period and stimulated lysozyme activity after two weeks, but not respiratory burst activity of phagocytic blood cells, total protein or total immunoglobulin in plasma. Both herbs when used alone or in combination increased the survival of fish after challenge with A. hydrophila. Thus, it can be concluded that the herb extracts added to diets act as immunostimulants and appear to improve the immune status and disease resistance of fish.

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